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A role for RNA helicase A in post-transcriptional regulation of HIV type 1

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ABSTRACT **Retroviruses must bypass the tight coupling of splicing and nuclear export of mRNA in their replication cycle because unspliced genomic RNA and incompletely spliced mRNA must be exported to the cytoplasm for packaging or translation. This process is mediated by a cis-acting constitutive transport element (CTE) for simple retroviruses and by the trans-acting viral protein Rev in concert with its response element (RRE) for complex retroviruses (e.g., HIV). Recently, we identified RNA helicase A (RHA) as a potential cellular cofactor for CTE. Here, we report that RHA also plays a role in Rev/RRE-mediated gene expression and HIV replication. RHA binds weakly to HIV-1 RRE independently of Rev. Overexpression of RHA, but not of an RHA mutant lacking helicase activity, increased both Rev/RRE- and CTE-dependent gene expression and the levels of unspliced HIV mRNA. Microinjection of antibodies to RHA into nuclei dramatically inhibited both CTE- and Rev-dependent gene expression in human cells. Exogenous RHA cDNA, but not the mutant RHA, rescued this inhibition. We propose that RHA is required to release both CTE- and RRE-containing mRNA from spliceosomes before completion of splicing, thus freeing them for nuclear export.**

HIV uses complex regulatory mechanisms to control gene expression. Such mechanisms involve the interdigitation of viral and cellular elements. Rev (regulator of virion protein expression) is a trans-acting viral protein that recognizes a cis-acting RNA element, the Rev response element (RRE), on the viral genome (see ref. 1 for review). Extensive studies to date revealed that Rev/RRE interaction facilitates the nuclear export of unspliced or singly spliced viral mRNA (2–4). However, Rev binding to RRE alone is insufficient. An activation domain on Rev, distinct from the RRE binding domain, is essential for function, presumably through binding of cellular effector molecules (2–6). This activator domain comprises a nuclear export signal (NES), which can be replaced functionally by the NES of some known export proteins (7–9). Several cellular proteins reportedly bind specifically to the Rev NES, including a nucleoporin-like protein called Rev activation domain-binding protein (10) or human Rev interacting protein (11), and the protein eIF-5A (12). More recently, Rev-NES was found to bind to the nuclear export receptor CRM-1/exportin-1 (13, 14), and it is likely that this interaction bridges the indirect binding of Rev-NES to Rev activation domain-binding protein/human Rev interacting protein (15, 16). Cellular proteins involved in RNA splicing/processing also have been found to bind directly to RRE (17) or the Rev/RRE complex (18). These proteins repressed Rev activity when overexpressed in cells. Other complex retroviruses, such as human T-cell leukemia virus and animal lentiviruses, also encode Rev-like proteins for post-transcriptional regulation (19).

In contrast to complex retroviruses, simple retroviruses, such as the Mason-Pfizer monkey virus, do not encode a Rev-like protein, even though there is a similar need for exporting unspliced genomic RNA to the cytoplasm for expression of viral structural proteins and for packaging. Instead, they use a cis-acting sequence, termed the constitutive transport element (CTE) (20, 21). CTE is able to substitute for Rev-RRE both in subgenomic gene expression constructs and infectious viral DNA clones. CTE or CTE-like elements have been described for other simple retroviruses—e.g., Rous Sarcoma virus (22)—as well as other viruses, such as the hepatitis B virus (23).

Recently, researchers in our group identified RNA helicase A (RHA) as a cellular factor that binds to functional CTE both *in vitro* and *in vivo* and shuttles between the nucleus and cytoplasm (24). In this paper, we show that RHA plays a role in both CTE- and Rev/RRE-mediated gene expression as well as HIV replication. We propose that RHA is required to release both CTE- and RRE-containing mRNA from spliceosomes before completion of splicing, thus freeing them for nuclear export.

MATERIALS AND METHODS

Cells and Plasmids. HeLa cells were maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum. Spinner HeLa cells were cultured in Joklik modified Eagle medium supplemented with 3.75% fetal bovine serum and 3.75% fetal calf serum.

Plasmids pDM128, pCMV128, and p121Rev were gifts from T. Hope (Salk Institute, La Jolla, CA). pDM128 and pCMV128 were RRE-containing chloramphenicol acetyltransferase (CAT) reporters with simian virus 40 (SV40) promoter and cytomegalovirus (CMV) promoter, respectively. p121Rev expresses the Rev protein of HIV-1. pHIV-Nhe is a chimeric HIV-1 HXB2 [gift of R. C. Gallo (University of Maryland)] used essentially as a wild-type HIV-1 in this study. pNL43R$^-$R$^-$S was generously provided by B. Felber (Frederick Cancer Research Facility, Frederick, MD). It contains an HIV-1 provirus with its RRE replaced by the SRV-1 CTE. pLT contains the full length RRE from HIV-HXB2 (17) and was used to synthesize *in vitro* biotin-labeled RRE RNA. pCMV110 was a plasmid expressing β-galactosidase under CMV promoter. It was used for normalizing transfection in subsequent assays. pcRHA was constructed by subcloning the

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Abbreviations: RRE, Rev response element; NES, nuclear export signal; CTE, constitutive transport element; RHA, RNA helicase A; RT, reverse transcription; SV40, simian virus 40; CMV, cytomegalovirus.

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full length RHA gene (a gift from J. Hurwitz, Memorial Sloan-Kettering Cancer Center, New York) into pcDNA3. The mutant RHA (mtRHA), which lacks the helicase activity, was kindly provided by M. Montminy (Harvard Medical School, Boston). It then was subcloned into pcDNA3. All of the plasmids were prepared with cesium chloride gradients.

Affinity Selection of RHA with RRE. HeLa nuclear extract was prepared from spinner cultures as described by Dignam *et al.* (25). Biotin-labeled RNAs were synthesized with Ambion's MEGAshortscript kit (Ambion, Austin, TX). The molar ratio of Biotin-rUTP (CLONTECH) to rUTP in the reaction was 1 to 5.67. Selection of RRE-binding nuclear proteins was carried out as described (26) with slight modification. In brief, 50 μl of streptavidin-conjugated agarose beads were washed twice with 500 μl of 1× FSP buffer (20 mM Tris·HCl, pH7.8/60 mM KCl/2.5 mM EDTA/0.1% Triton X-100) and were resuspended in 50 μl of 1× FSP plus 2.5 μl of yeast tRNA (10 mg/ml, Sigma). While the beads were kept on ice, 200 μl of nuclear extract were mixed with 70 μg of biotinylated RNA and 500 μg of yeast tRNA. The volume was adjusted to 500 μl with 101 FSP (final 1×) and dH_2O. After incubating on ice for 10 minutes, the total volume was brought to 1 ml with FSP buffer, and the salt concentration was adjusted to 350 mM with 2 M KCl. The sample was transferred to the tube with agarose beads and was rotated overnight at 4°C. The beads were washed three times with 1× WB350 buffer (20 mM Tris·Cl, pH7.8/350 mM KCl/0.01% Nonidet P-40) and once with 1× FSP. Proteins were eluted by boiling the beads in protein sample buffer and were separated on a 10% SDS/PAGE gel. For immunodetection of RHA, proteins were transferred to poly(vinylidene difluoride) membrane with standard procedure (27).

Immunodetection of RHA. After transfer, the membrane was blocked for 1 hr with 5% nonfat dry milk in TBS buffer (150 mM NaCl/10 mM Tris·Cl, pH8.0/0.05–0.1% Tween-20). Then, first antibody (rabbit anti-RHA serum was generously provided by J. Hurwitz) was added with 1:5,000 dilution in 5% nonfat dry milk. After 1-hr incubation at room temperature, the membrane was rinsed twice with TBS and was washed for 15 min once and 5 min twice. Then, the membrane was incubated in second antibody (horseradish peroxidase conjugated goat anti-rabbit antibody, 1:5,000 dilution in 5% nonfat dry milk) for 1 hr at room temperature, followed by rinsing and washing as described above. To view the RHA band on membrane, the blot was developed with enhanced chemiluminescence solutions (Amersham) and was exposed to x-ray film.

Immunoprecipitation and Reverse Transcription (RT)–PCR. HeLa cells in 100-mm dishes were transfected with either pCMV128 (1.6 μg) plus p121Rev (3.6 μg) or pCMV128 (1.6 μg) plus pcDNA3 (3.6 μg). Forty-eight hours later, cells were lysed in 1 ml of 0.65% Nonidet P-40 lysis buffer (150 mM NaCl/10 mM Tris·Cl, pH7.8/1.5 mM $MgCl_2$/0.65% Nonidet P-40). Cell lysates were precleared with normal rabbit serum by incubating overnight with 20 μl of normal rabbit serum conjugated agarose beads at 4°C. The resulting lysate was divided into three parts. Each part was mixed with 40 μl of protein-A agarose beads (Pierce) plus 2 μl of one of the following: (*i*) normal rabbit serum, (*ii*) rabbit anti-RHA serum, or (*iii*) rabbit anti-Rev serum. After incubating at 4°C overnight, the beads were washed three times with lysis buffer. RNA was extracted from the beads with Ultraspec (Biotecx Laboratories, Houston). RT-PCR was carried out with RRE-specific primers, and the PCR products were visualized in agarose gels under UV light.

p24 Assay. HeLa cells were cotransfected with either pHIV-Nhe or pNL43R^-R^-S plus 10-fold excess in micrograms of pcRHA or pcDNA3. In a dose–response experiment, various amount of pcRHA was used. Starting from 2 days after transfection, culture medium was collected every day for 3 days, and the amount of p24 in the medium was measured with a standard p24 kit (Coulter).

Microinjection. Microinjection analysis was performed essentially as described (28). Before the injection, primary HS68 human fibroblasts routinely were rendered quiescent by incubation in serum-free medium for 24–36 hr. Microinjection experiments were performed on coverslips. Each data point is derived from three separate experiments conducted on different days, involving at least 250 injected cells each time. The following plasmids were injected into the nuclei of cells at a concentration of 100 μg/ml: pCM228, a modified version of the RRE-containing plasmid pDM128, with a CMV promoter and LacZ reporter gene; pCM238CTE with CTE substituting for RRE in pCM228; pRev; CMV-LacZ; and SV40LacZ. A polyclonal antibody against native RHA (provided by J. Hurwitz) was injected at a concentration of 20 mg/ml. In all cases where no specific antibody was used in the experiment, preimmune rabbit IgG was coinjected, allowing the unambiguous identification of the injected cells in addition to serving as a preimmune control for the experiment. Expression of reporter gene was allowed to proceed overnight before fixation for staining. β-Galactosidase activity was detected by incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Injected cells were identified by staining with tetramethylrhodamine-conjugated secondary antibodies. In cells expressing high levels of β-galactosidase, the blue staining tended to quench rhodamine fluorescence. For this reason, injected cells

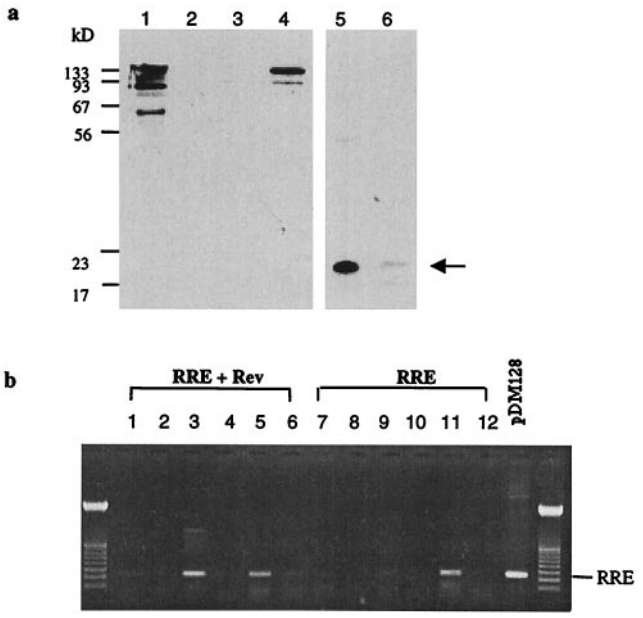

FIG. 1. Interaction between RHA and RRE. (*a*) *In vitro* binding of RHA to CTE but not to RRE in the presence or absence of Rev. RNA Selection with biotin-labeled RRE (lanes 2, 3, and 6) or CTE (lane 4) was carried out as described (13). Recombinant Rev was added to the nuclear extract in some samples (lanes 2, 5, and 6). Lanes 1–4 were probed with anti-RHA antibodies and lanes 5–6 were probed with anti-Rev antibodies. Lane 1 (nuclear extract) and lane 5 (Rev mixed with nuclear extract) were positive controls for direct immunodetection with antibodies without RNA selection. The arrow indicates the position of the Rev-specific band in lanes 5 and 6. (*b*) Detection of *in vivo* interactions between RHA and RRE or Rev-RRE by using immunoprecipitation and RT-PCR. Lysates from cells transfected with pRev and pRRE (lanes 1–6) or pRRE alone (lanes 7–12) were immunoprecipitated with normal serum (lanes 1, 2, 7, and 8), anti-Rev antibodies (lane, 3, 4, 9, and 10), or anti-RHA antibodies (lanes 5, 6, 11, and 12). Half of the RNA obtained from the precipitate was subjected to RT (lanes 1, 3, 5, 7, 9, and 11) and PCR amplification using RRE-specific primers. The other half was processed under the same conditions without RT (lanes 2, 4, 6, 8, 10, and 12). The expected product for RRE was 230 bp in length.

were counted as those exhibiting either nuclear rhodamine fluorescence, blue X-gal staining, or both. All cells showing any trace of blue staining were scored as positive for expression to avoid any possible subjectivity in the analysis. Experiments were analyzed, and photography was performed, on a Zeiss Axiophot epifluorescence microscope.

Northern Blot Analyses. HeLa cells were transfected with pHIV-Nhe plus either pcRHA or pcDNA3. Two days later, total cellular RNA was extracted with Ultraspec. RNA (5 μg) was run in agarose gel and was blotted onto GeneScreen Plus membrane (NEN). The membrane then was probed with ^{32}P labeled HIV-1 LTR (RU5 region) and was exposed to x-ray film.

RESULTS

Binding of RHA to RRE or the RRE-Rev Complex. Because RHA specifically binds to functional CTE *in vitro* and *in vivo* (24), we wanted to determine whether it also binds to RRE or the RRE-Rev complex. HeLa nuclear extract was incubated with biotin-labeled CTE RNA, biotin-labeled RRE RNA, or a combination of biotin-labeled RRE RNA and recombinant Rev protein. Proteins bound to the RNA were pulled down with streptavidin beads, were eluted and resolved on SDS/PAGE gels, and were probed with a polyclonal antibody against RHA. Although CTE specifically selected RHA (Fig. 1*a*, lane 4), neither RRE alone nor RRE plus Rev brought down RHA (Fig. 1*a*, lanes 2 and 3), suggesting that, under these conditions, interaction between Rev/RRE and RHA is either weak or nonexistent. Total nuclear extract immunoprecipitated with anti-RHA was included as a positive control (Fig. 1*a*, lane 1). In contrast, biotinylated RRE RNA could specifically pull down Rev from a mixture of recombinant Rev protein and nuclear extract (Fig. 1*a*, lane 6).

We then investigated the potential *in vivo* interaction between RRE and RHA. Anti-RHA antibodies were used to immunoprecipitate interacting protein and nucleic acid components from HeLa cells that were transfected with pDM128 (an RRE-containing chloramphenicol acetyltransferase reporter plasmid) or pDM128 plus pRev. RNA was extracted from these complexes and was subjected to RT-PCR analyses with RRE-specific primers. Fig. 1*b* shows that anti-RHA brought down RRE-containing RNA in the presence (lane 5) or absence (lane 11) of Rev. Normal rabbit sera and anti-Rev antibodies were used as negative and positive controls, respectively. We also used a construct that lacks RRE as a negative control and showed that RRE RNA was preferentially brought down compared with the RRE-negative RNA transcript (data not shown). These results suggest that RHA may interact with RRE *in vivo* in a Rev-independent manner. It is possible that

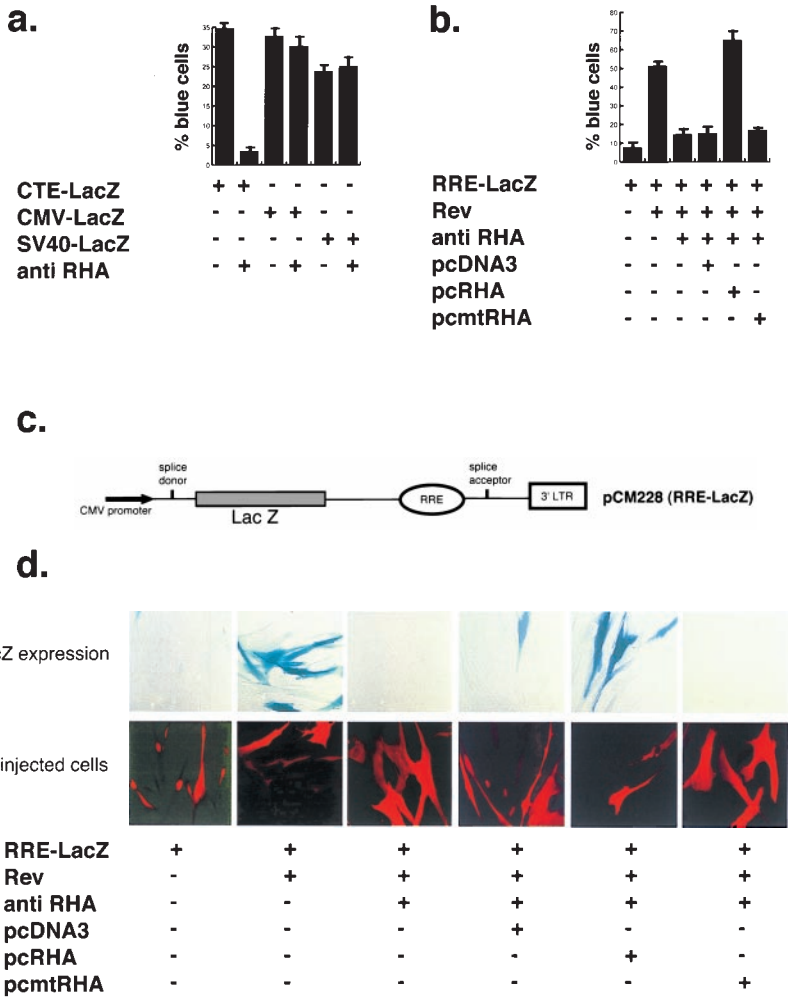

FIG. 2. Microinjection of anti-RHA antibody blocks CTE or Rev/RRE-mediated reporter gene expression in human cells. (*a*) HS68 fibroblasts were injected with LacZ reporter constructs in which expression of β-galactosidase either was mediated by CTE or was driven constitutively by viral promoters. Constructs were coinjected with either preimmune IgG or anti-RHA as indicated. (*b*) Microinjection of a Rev-dependent RRE reporter construct with preimmune IgG or anti-RHA. Rescue experiments were conducted by coinjection of constructs encoding either wild-type RHA or an enzymatically inactive point mutant, mtRHA (14). The error bars represent standard errors. (*c*) Schematic representation of RRE reporter construct. (*d*) Phase contrast and corresponding immunofluorescence photomicrographs that demonstrated typical experimental results.

this interaction is bridged by other cellular proteins or nucleic acids *in vivo*.

Inhibition of RRE/Rev-Mediated Gene Expression by Anti-RHA Antibodies. In cotransfection experiments, we consistently observed a 2-fold increase in Rev- or CTE-mediated reporter gene expression by pcRHA whereas gene expression driven by the HIV-1 LTR was not affected (data not shown). An RHA mutant (mtRHA) containing a single amino acid substitution in the helicase domain that abolishes its ATP-binding and helicase activity (29) did not exert this effect (data not shown). This modest level of activation may belie a critical role for RHA in CTE and Rev function because the endogenous level of RHA may already approach the threshold for optimal activity. Therefore, we examined the effect of antibodies to RHA on both CTE and Rev function *in vivo*. The high-titer, monospecific, polyclonal RHA antibodies used in these studies were raised in rabbits against native RHA (30). These were co-microinjected into the nuclei of primary HS68 human fibroblasts with a CTE-containing LacZ reporter plasmid, an RRE-containing LacZ reporter plasmid alone, or in combination with pRev. Preimmune rabbit IgG was injected in parallel and served both as a marker for injected cells not receiving anti-RHA and as a nonimmune control. CMV-lacZ or SV40-lacZ plasmids were used as negative controls for the reporter plasmids (28, 31). After overnight incubation, cells were stained and examined under an epifluorescence microscope. β-galactosidase activity was detected by incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal); injected cells were identified by staining with tetramethylrhodamine-conjugated secondary antibodies directed against the coinjected rabbit IgG. As shown in Fig. 2*a*, anti-RHA inhibited CTE-mediated LacZ expression by ≈85% but had no effect on lacZ expression from the CMV or SV40 promoted constructs. Anti-RHA also inhibited Rev-mediated LacZ expression to approximately the basal level seen in the absence of pRev. This inhibition was rescued when a plasmid expressing wild-type RHA cDNA (pcRHA) was coinjected but not when pcmtRHA or pcDNA3 was used (Fig. 2 *b–d*). These results indicate that RHA plays a critical role in both CTE and Rev function.

RHA Increased Viral p24 Expression. We further investigated whether RHA may play a role in regulating virus expression. HeLa cells were transfected with an infectious HIV-1 provirus clone (pHIV-Nhe) or an RRE(−)/Rev(−) HIV-1 provirus clone that contained CTE in the Nef reading frame (pNL43R$^-$R$^-$S) (21). Various amounts of pcRHA were cotransfected with the viral DNA. Culture media were collected daily for p24 assays. Fig. 3 *a* and *b* shows that, on day 4 after transfection, cells that received RHA produced greater levels of p24 than those transfected with pcDNA3. Similar results were obtained for both wild-type HIV-1 and the CTE-containing HIV (Fig. 3*a*). We also showed that the increase in expression of p24 in response to RHA was dose-dependent (Fig. 3*b*).

RHA Regulates Virus Expression at the Post-Transcriptional Level. To determine the mechanism of increased viral replication in response to RHA overexpression, we isolated total RNA from HeLa cells transfected with pHIV-Nhe plus

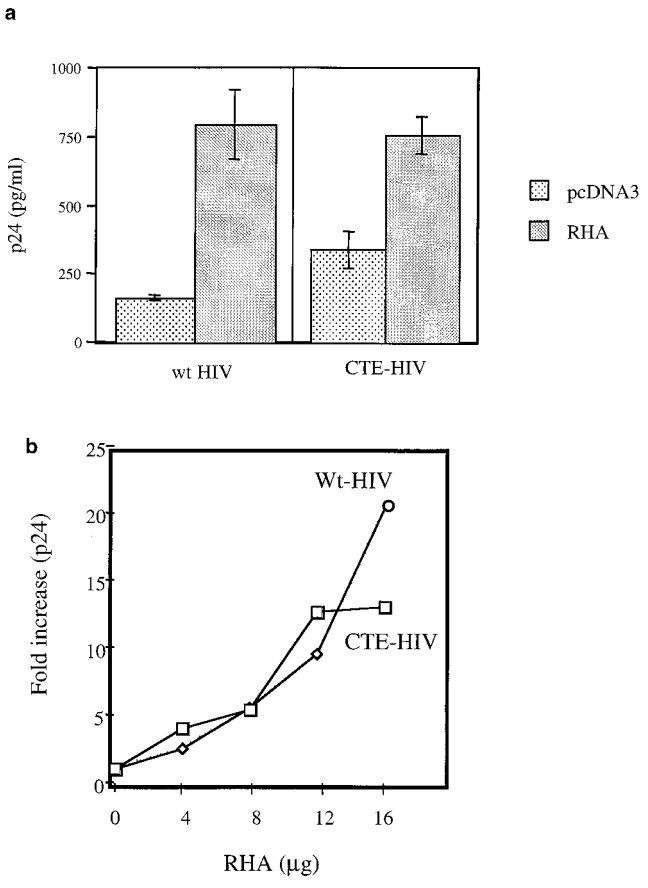

FIG. 3. Increase of p24 production in virus producing cells cotransfected with RHA. (*a*) RHA increased the p24 production of HIV. HeLa cells were transfected with a plasmid containing wild-type HIV provirus or a CTE-containing modified HIV provirus (pNL43R-R-5). About 10× more (in milligrams) pcRHA or pcDNA3 was included in the transfection. p24 was measured on day 4 after transfection. The error bars indicate the range of values from two different experiments, and the average values were used in the graphs. (*b*) RHA dose-dependent increase of p24 production. HeLa cells were cotransfected with wild-type or CTE-modified HIV plus various amount of pcRHA. pcDNA3 was included to keep the total amount of DNA balanced at each point. p24 in culture medium was measured on day 4 after transfection.

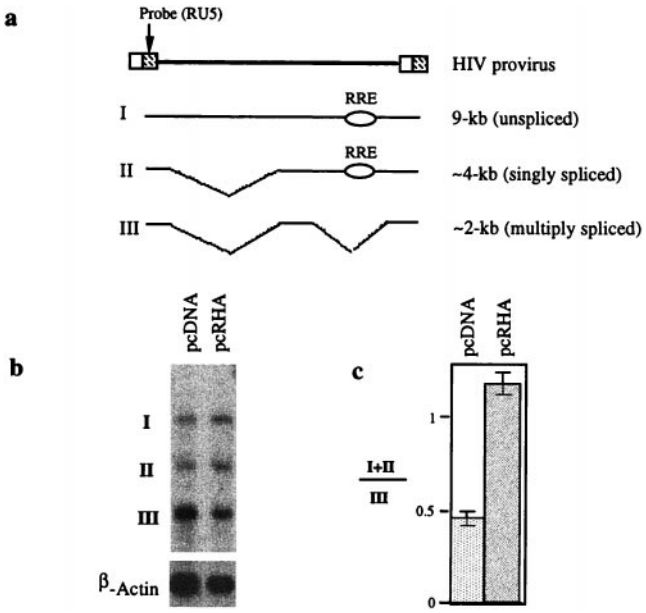

FIG. 4. RHA enhanced the production of unspliced or singly spliced HIV RNA. (*a*) Structure of the three species of viral RNA. (*b*) Northern blot analysis of HIV RNA in cells cotransfected with HIV proviral DNA and pcRHA or pcDNA3. HeLa cells were transfected as described in Fig. 3*A*. Two days after transfection, total cellular RNA was extracted and subjected to Northern blot analysis using the RU5 sequence of HIV-1 LTR as probe. I, II, and III represent unspliced, singly spliced, and multiply spliced viral RNA, respectively. (*c*) Increased ratio of unspliced and singly spliced viral RNA to multiply spliced RNA in cells transfected with pcRHA. The amount of each viral RNA species was quantified by measuring the band intensity on the x-ray film by using image analysis software (IMAGEQUANT, Modular Dynamics).

either pcRHA or pcDNA3 (pHIV:pcRHA = 1:10) and subjected them to Northern blot hybridization with an LTR-specific probe. Fig. 4*b* shows a representative Northern blot result, clearly indicating that overexpression of RHA increased the ratio of unspliced and singly spliced mRNA (species I and species II) to multiply spliced mRNA (species III) but did not increase the steady-state levels of total viral mRNA. Fig. 4*c* shows the average value of two independent transfection experiments. These results indicate that regulation of HIV expression by RHA occurs at the post-transcriptional level.

DISCUSSION

Several lines of evidence have indicated that CTE and Rev/RRE use distinct nuclear export pathways. In the *Xenopus* oocyte system, several investigators showed that Rev-NES peptide conjugates competed with nuclear export of RRE-containing RNA in the presence of Rev but had no effect on CTE export (32, 33). Likewise, excess CTE RNA inhibited its own export but not that of RRE. Furthermore, the interaction of Rev with CRM-1, and hence Rev-mediated nuclear export of RRE-containing RNA, is sensitive to leptomycin B (34) whereas the CTE-mediated gene expression was leptomycin B insensitive (T. Hope, personal communication). Recently, a bi-directional nuclear transport domain at the carboxyl-terminus of RHA, which was also refractory to leptomycin B inhibition, was identified (H.T., T. Hope, T. Middlesworth, and F.W.-S., unpublished results). These results, along with our observations that RHA binds strongly to functional CTE, but not to RRE, are consistent with RHA playing a role in the nuclear export of CTE-RNA but not RRE-RNA. CTE also binds to TAP, the human homolog of Mex67p, a yeast protein that has been localized to the nuclear pore complex (35). There is no evidence that TAP is a shuttle protein, however, and TAP and RHA may play complementary roles in CTE-RNA export.

Surprisingly, our results presented here indicate that RHA also plays an important, positive role in Rev regulation. Although RHA was reported to increase transcription from CBP targeted promoters (29), its activation of HIV expression is apparently at the post-transcriptional level, specifically, in the increase of unspliced and singly spliced mRNA (Fig. 4). It is also intriguing that mtRHA, which lacks helicase activity, was unable either to increase Rev activity or to rescue inhibition of Rev by anti-RHA antibodies. These observations suggest that the helicase activity of RHA is essential for CTE and Rev function.

RNA helicases have been implicated in splicing and nuclear export of mRNA in diverse species. The yeast splicing factor, Prp22, as well as its human homologue, human RNA helicase 1, have been postulated to promote the release of spliced mRNA from the spliceosome (36). A negative dominant mutant of human RNA helicase 1, with the same mutation as mtRHA in the conserved ATP-binding site of RNA helicases, specifically inhibited the release of spliced mRNA from spliceosomes *in vitro* (37). We propose a model for the role of RHA in the post-transcriptional regulation of retroviral mRNA as follows. Like all cellular pre-mRNA, CTE- and RRE-containing mRNAs are engaged in the assembly of spliceosomes concomitantly with transcription. Although many different helicases are involved in forming and dissolving RNA interactions throughout the splicing process (38), it is likely that a specific subset of helicases are recruited to the spliceosome complex only when splicing is complete, possibly through an interaction with specific splicing factors. We propose that this subset includes human RNA helicase 1 and RHA and that direct interaction of RHA with CTE- and RRE-containing RNAs promotes the premature release of these RNA without initiating or completing the splicing process. In the case of CTE-RNA, RHA, using its own export signal sequences, may further act as a chaperone for the RNA to transit the nuclear pores. In contrast, the interaction of RHA with RRE may not be strong or stable enough to complete the export process, thus requiring Rev to bind RRE. Thus, our model assigns a role for RHA common to both simple and complex retroviruses, at a step (release from spliceosomes) upstream from the actual export step of this pathway. Other cellular factors may be involved in nonoverlapping steps of the two export pathways: e.g., CRM-1 for Rev/RRE and TAP for CTE (35).

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